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13. ABSTRACT (Maximum 200

Overexpression of activated c-Src leads to increased invasive behavior in MDA-MB-231 cells. Localized matrix degradation at invadopodia was increased more than 4 fold. Zymogram analysis of conditioned media from these cells demonstrated that the level of the extracellular matrix degrading metalloproteinase, MMP-9 was dramatically up regulated in transfected cells expressing constitutively activated c-Src (Y527F). Conversely, expression levels of MMP-9 in transfected cells expressing the dominant negative, kinase inactive c-Src (K295R) were suppressed. Matrix degradation could be inhibited using a monoclonal antibody that blocked the enzyme activity of MMP-9. These results suggest that membrane-associated MMP-9 was largely responsible for the localized matrix degradation observed at invadopodia in cells over-expressing kinase activated c-Src. Subcellular fractionation confirmed the localization of MMP-9 to invadopodia-enriched fractions. Interestingly, activated MMP-2 was also present in these fractions. Co-immunoprecipitation, Western blotting and zymography demonstrated that MMP-9 is localized at invadopodia via an interaction with the cell surface receptor, $\alpha 3$ integrin. Levels of integrin expression were not modulated by c-Src transfections. We conclude that c-Src activation promotes invasion, in part by increasing the expression of MMP-9, which associates with $\alpha 3$ integrin to promote localized matrix degradation at invadopodia.

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Proposal Title: Integrin mediated invasion in breast cancer

Postdoctoral Fellow: Dr. Emma T. Bowden
Research Mentor: Dr. Susette Mueller
Key Words: MMP-9, activation, integrin, invasion, degradation

Introduction

Metastasis remains the primary factor in the morbidity of human breast cancer, since advances in the detection and removal of primary lesions have not been matched by the development of successful therapies for disseminated disease. During metastatic progression, tumor cells encounter numerous extracellular matrices and basement membranes as they leave the site of primary tumor growth. Thus the ability to degrade a range of the components that comprise these structures is advantageous to the dissemination of tumor cells (Liotta et al. 1991). There is a body of literature suggesting that the cell surface integrins, which act as receptors for many of these basement membrane and extracellular matrix molecules will also indirectly regulate the synthesis of the proteases responsible for extracellular matrix degradation. For example, MMP-9 is an extracellular matrix degrading protease and one of a class of proteases that has been implicated in several stages of tumor progression including local invasion, loss of basement membrane integrity, angiogenesis and ultimately metastatic tumor progression (Bernhard et al. 1994; Matrisian, 1990; Nakajima et al. 1993; Sehgal et al. 1998). Elevated levels of MMP-9 expression have been detected in a number of human cancers, including lung, bladder and breast (Bianco et al. 1998; Iizasa et al. 1999; Rha et al. 1997). *In vitro*, cellular transformation can initiate inappropriate levels of MMP-9 expression and this represents a major contribution to neoplastic transformation (Sato et al. 1993). This study addressed the hypothesis that the kinase activity of c-Src stimulated by integrins leads to increased MMP-9 expression and therefore increased invasive potential.

Training and Research Accomplishments

Task 1: Identify the effects of c-Src overexpression on MMP expression levels and activity. Correlate these effects with the increased membrane associated matrix degradation.

        Signaling events leading to the activation of NFκB, SP-1, Ets, AP-1 and the retinoblastoma binding factors in tumor cells can all contribute to increased expression of MMP-9 by transcriptional activation (Sato et al. 1993). These signals can be initiated by v-Src transformation, a number of growth factor and integrin mediated signaling pathways (Ellerbroek et al. 1998; Khan, Falcone, 1997; Kondapaka et al. 1997; Sato et al. 1993). We hypothesized that overexpression of constitutively activated c-Src (Y527F) would cause similar a transcriptional upregulation of MMP-9. As a model system we used transfected MDA-MB-231 cells over-expressing either wild-type c-Src, kinase-inactive "dead" c-Src (K295R) or constitutively activated c-Src (Y527F) constructs to examine the role of c-Src kinase activity on *in vitro* invasion. These mutations of c-Src have been previously characterized for their kinase activity and transforming potential (Bagrodia et al. 1991; Kmiecik, Shalloway, 1987).

        Dr. Toshiyuki Yoneda generously provided the c-Src transfected cells (Myoui et al. 1996). We used Western blotting to confirm that the transfected cell lines overexpressed c-Src (appendix 4, supplemental figure 1). We used immunoprecipitation and Western blotting to obtain supporting data for the enhanced kinase activity of the c-Src (Y527F) mutant and suppressed kinase activity of the c-Src (K295R) mutant transfected cells (appendix 4, supplemental figure 2). Since cortactin is a well described c-Src substrate, and increased phosphorylation of this cytoskeletal molecule has been described in response to c-Src mediated integrin stimulation, we examined the levels of cortactin tyrosine phosphorylation as an indication of the kinase activity of c-Src in the transfected cell lines. Western blotting of equal amounts of cell lysates from the parental cell line and the transfected cell lines demonstrated that overexpression of any of the c-Src constructs did not affect the levels of expression of cortactin (appendix 4, supplemental figure 2a). However, if tyrosine phosphorylated proteins were immunoprecipitated from each of these cell lines and run on a western blot, a highly tyrosine phosphorylated protein corresponding to the molecular weight of cortactin was disproportionately represented in the c-Src (Y527F) constitutively activated cell line (appendix 4, supplemental figure 2b). This Western blot was stripped and reprobed with antibodies against cortactin to confirm the identity of this protein (appendix 4, supplemental figure 2c). The hyperphosphorylation of cortactin supported the presence of activated c-Src in this cell line. Conversely, we also saw a decrease in the levels of tyrosine phosphorylated cortactin in the kinase-dead c-Src (K295R) transfected cells.

        As previously described, when invasive MDA-MB-231 breast cancer cells are cultured on FITC-gelatin, the cells produce dark holes in a background of bright fluorescent matrix (Bowden et al. 1999; Coopman et al. 1998)(appendix 4, supplemental figure 3). These dark holes are produced by membrane protrusions with extracellular matrix degrading capabilities that extend from the ventral surface of the cell. These

invasive protrusions have been termed "invadopodia" and their formation requires a coordination of cytoskeletal rearrangements along with the localization and activation of certain cell surface associated proteases (Monsky et al. 1993; Monsky et al. 1994; Mueller et al. 1992; Nakahara et al. 1996).

Previously, we have compared the invasive capacity of breast cancer cells with their invadopodia activity(Coopman et al. 1998). We have also demonstrated that behavior in this assay correlates well with invasive potential as measured in other well-characterized *in vitro* invasion assays (Coopman et al. 1998). We demonstrated that overexpression of activated c-Src (Y527F) produced a significant increase in sites of local invasion over either the parental cell line or the kinase inactive c-Src (K295R) over-expressing cells (appendix 4, supplemental figure 3; appendix 3, manuscript figure 2).

Gelatin zymography revealed that the parental, kinase "dead" c-Src (K295R) and kinase activated c-Src (Y527F) cell lines all expressed both MMP-9 and MMP-2 (appendix 3, manuscript figure 1a). However, quantification revealed that overexpression of kinase-activated c-Src (Y527F) led to an approximately four-fold upregulation of expression of MMP-9 over the levels expressed in the parental cell line (appendix 3, manuscript figure 1b). In contrast overexpression of kinase-inactive c-Src (K295R) did not increase expression levels of MMP-9. In fact, we saw inhibition of the levels of MMP-9 produced by the c-Src (K295R) cell lines. This suggested that the kinase activity of c-Src was responsible for the increased levels of MMP-9 expression in the c-Src (Y527F) transfectants. Levels of MMP-2 expression remained unaltered in each of the cell lines examined.

Since we hypothesize that cell surface recruitment of extracellular matrix degrading proteases is a critical component in the acquisition of the ability to invade and metastasize we were particularly interested in the subcellular distribution of MMP-9. In particular we wanted to examine the possibility that MMP-9, recruited to invadopodial membranes was mediating the increased invadopodia activity measured in the c-Src (Y527F) transfectants. To this end we utilized a subcellular fractionation protocol that allows us to isolate an enriched invadopodial membrane fraction (Bowden et al. 1999). We concentrated the gelatinases from each fraction using Gelatin Sepharose and analyzed them by gelatin zymography. Using this technique we verified that MMP-9 was enriched in the invadopodia fraction of c-Src (Y527F) transfected cells (appendix 3, manuscript figure 4). A single band of 92 kDa suggested that pro-MMP-9 was present in this fraction. We also saw an enrichment of activated MMP-2 in the invadopodia fraction of both the parental and c-Src (Y527F) transfected cells. Activated MMP-2 appeared exclusively in the invadopodia fraction of the parental cells. We saw some activated MMP-2 in the cell body membrane fraction of the c-Src (Y527F) transfected cells. However, activated MMP-2 appeared predominantly in the invadopodia fraction. Pro-MMP-2 appeared in all of the fractions and lysates from these cell lines.

Task 2: Determine the contribution/requirement of MMP-2 and MMP-9 in localized membrane associated degradation.

We wanted to further examine the possibility that the increased level of MMP-9 expression was responsible for the observed increase in invadopodia activity seen in the c-Src (Y527F) transfected cells. The commercially available anti-MMP-9 mAb Ab-1 (Chemicon) has been demonstrated to inhibit MMP-9 protease activity (Ramos-DeSimone et al. 1999; Ramos-DeSimone et al. 1993). We incorporated this antibody at increasing doses into our invadopodia assay adding it to both the growth media and the gelatin matrix. We demonstrated a dose dependent inhibition of matrix degradation for the c-Src (Y527F) transfectants (appendix 3, manuscript figure 3). At the highest dose of antibody examined (100µg/ml) there was a ~65% inhibition of invadopodia activity. It should also be noted that an irrelevant antibody of the same isotype as the experimental anti-MMP-9 antibody did not inhibit invadopodia activity of the c-Src (Y527F) transfected cells. The successful inhibition of invadopodia activity using specific anti-MMP-9 antibodies clearly demonstrated that MMP-9 was participating in the increased invadopodia activity measured in the c-Src (Y527F) transfected cells.

Interestingly, in an initial repetition of the invadopodia inhibition using anti-MMP-9 antibodies, an anti-MMP-2 antibody was chosen as a control antibody. This antibody had not previously been described as having any enzyme inhibitory activity. However, when used at a 100µg/ml as a negative control for the equivalent dose of MMP-9 inhibitory antibody we saw a significant increase in the measured invadopodia activity. To confirm this unexpected result we repeated the experiment using a dose response of antibody (appendix 4, supplemental figure 4). Surprisingly there was a dose-dependant increase in invadopodia activity. There is a complicated relationship between MMP family members and their endogenous inhibitors (tissue inhibitors of metalloproteinases or TIMPs) which are also produced by the cell lines in question. It is possible that the anti-MMP-2 antibody interfered with binding of TIMP1 and TIMP2 to pro-MMP-2 releasing free pro-MMP-2 available for interaction with the cell surface and activation. Experiments to determine if the increased invadopodia activity observed in this experiment were mediated by MMP-2 activity were deemed beyond the scope of the approved statement of work.

Experiments using TIMP1 and TIMP-2 were included in the statement of work specifically to address the contribution of MMP-9 in the measured invadopodia activity. These experiments were not performed because we demonstrated that both MMP-2 and MMP-9 were enriched in the invadopodia fraction of the c-Src (Y527F) transfected cells (appendix 3, manuscript figure 4). TIMP-1 and TIMP-2 inhibit both MMP-2 and MMP-9, eliminating the possibility of distinguishing their individual contributions to the measured increase in invadopodia activity.

Task 3: Examine the c0-association of α3β1 integrin and MMP-9 in the highly invasive c-Src (Y527F) transfected MDA-MB-231 cells

We wanted to examine the possibility that there was a cell surface receptor for MMP-9 at invadopodia. A number of transmembrane molecules have been localized to invadopodia including several integrin subunits and the activator for MMP-2, MT1-MMP. There is precedent in the literature for interactions between integrins and proteases (Brooks et al. 1996; Mueller et al. 1999). Therefore, we examined the possibility that one of the integrin subunits localized to invadopodia was coupling MMP-9 activity to the membrane.

MMP-9 has previously been colocalized with β1 integrin at endothelial cell contacts and isolated from shed plasma membrane vesicles enriched with this integrin (Dolo et al. 1998; Partridge et al. 1997). We have previously demonstrated the localization of α3β1 integrin to invadopodia in MDA-MB-231 cells (Coopman et al. 1996). This integrin heterodimer is the major one expressed in this cell type and experiments using antibodies to perturb α3 integrin in this cell line proved to modulate invasive capacity (Coopman et al. 1996). Therefore, we hypothesized that the α3 integrin subunit was a potential MMP-9 receptor candidate.

Several experimental designs were employed to identify interactions between MMP-9 and the cell surface. Initially, the membrane insoluble, reversible crosslinking agent DTSS was employed to stabilize cell surface interactions. Immunoprecipitations and Western blotting allowed an association between MMP-9 and α3 integrin to be isolated from c-Src (Y527F) transfected cells (appendix 4, supplemental figure 5). There was no co-immunoprecipitation from either the parental, wild-type c-Src or the kinase-dead c-Src (K295R) transfected cells. Also there was no co-immunoprecipitation of this protein with either α2 or β1 integrin. However, the molecular weight of the protein band identified was about 20kDa too large for the described molecular weight of MMP-9. Cross-linking may have altered the electrophoretic mobility of MMP-9 under these experimental conditions. It was also possible that MMP-9 remained in a complex with some other unidentified protein and that reduction of the crosslinking did not resolve this association.

We confirmed the putative association of MMP-9 and α3 integrin under a second set of experimental conditions. Lysates were isolated without crosslinking and co-immunoprecipitations repeated. Co-immunoprecipitation and Western blotting again identified an association between α3 integrin and MMP-9 in the c-Src (Y527F) transfected cell lines. This association was specific to α3 integrin, it did not occur with α2 integrin (appendix 3, manuscript figure 5). Because none of the integrin antibodies used in these experiments will western blot under reducing conditions, it was critical to demonstrate that, under the conditions used for immunoprecipitation, that all the integrin subunits of interest would immunoprecipitate effectively. To demonstrate this, we biotinylated cell surface proteins before making cell lysates. Western blots were probed with streptavidin to detect the presence of immunoprecipitated integrin subunits (data not

shown). Using this technique we established that all the integrin subunits we examined were effectively immunoprecipitated under the experimental conditions examined.

By Western blotting we were only able to detect a single 92kDa MMP-9 moiety that immunoprecipitated with α3 integrin. However, the antibodies used in Western blotting only recognize latent MMP-9 under reducing conditions. Unfortunately there are no commercially available antibodies that will recognize activated MMP-9 under these conditions. To address the possibility that there were other activated forms of MMP-9 present, the co-immunoprecipitation experiment was duplicated and analyzed by zymography. This technique allows all the activated and non-activated forms of MMP-9 to be visualized. All members of the MMP family are secreted as proenzymes requiring activation, a critical point in the regulation of their activity, zymography allows both activated and non-activated forms of these proteases to be examined. When analyzed by zymography, α3 co-immunoprecipitations isolated several bands of gelatinolytic activity. One of these bands was the same molecular weight as the MMP-9 band identified by western blotting, however, there were also several gelatinolytic bands of lower molecular weight. Initially, it was assumed, because of their molecular weights, that the lower molecular weight bands identified corresponded to the presence of MMP-2. However, recently MMP-9 immunoprecipitations and zymography using lysates from the c-Src (Y527F) transfected cells confirmed the presence of low molecular weight bands of gelatinolytic activity (data not shown). To address the identity of this gelatinase first we confirmed that we had in hand an MMP-2 antibody that recognized all activated and non-activated forms MMP-2 by Western blotting. MMP-9 immunoprecipitations were examined by western blotting. After confirmation of an effective MMP-9 immunoprecipitation reaction, the Western blot was re-probed for the presence of MMP-2. This technique demonstrated the absence of MMP-2 from these immunoprecipitation reactions. Whilst this result does not confirm that the lower molecular weight bands identified by zymography in co-immunoprecipitates of α3 integrin are activated MMP-9, it suggests that they are not MMP-2. Further studies would be required to confirm the identity of these lower molecular weight bands.

Fractionation of c-Src (Y527F) to confirm the subcellular association of α3 integrin and MMP-9 at invadopodia was unsuccessful. Both crosslinking and non-crosslinking approaches were employed to overcome this technical problem. Fractionation and gelatin zymography suggested that the only membrane associated localization for MMP-9 is the invadopodia fraction (appendix 3, manuscript figure 4) and, we have previously localized α3 integrin to invadopodia (Coopman et al. 1996). However, this remains circumstantial evidence and the technical difficulties encountered trying to answer these questions have not been resolved.

Task 4:Determine the role of α3β1 integrin in c-Src activation, and the subsequent upregulation of cellular invasion and MMP-9-α3β1 association.

Integrins are transmembrane proteins consisting of α and β heterodimers. Each integrin has distinct ligand binding activity. These receptors mediate both cell adhesion and migration *in vitro* (for recent review see (Miyamoto et al. 1998)). Integrins have a

critical role in the transduction of signals from the extracellular matrix to the inside of the cell. Distinct roles for integrin heterodimers have been described in invasive tumor cell behavior. These include modification of migratory properties and regulation of invasive capacity (Brooks et al. 1996; Coopman et al. 1996; Lochter et al. 1999; Rabinovitz, Mercurio, 1997; Shaw et al. 1997).

We attempted to identify an integrin perturbing treatment that would cause c-Src activation in wild type c-Src transfected cells. We have already demonstrated using several techniques that the wild type c-Src transfected cells demonstrated similar levels of invadopodia activity as the parental untransfected cell line. We hoped to confirm the hypothesis that functional perturbation of $\alpha3\beta1$ integrin would stimulate c-Src activity resulting in upregulation of MMP-9 and increased invadopodia activity. Previous data had suggested that perturbation of $\alpha3\beta1$ integrin with either laminin peptides or anti-$\alpha3$ antibodies produced an increase in invasive behavior in untransfected MDA-MB-231 cells although the mechanism for this was not elucidated (Coopman et al. 1996)}.

One of the assays we had hoped to employ in this aspect of the study was the co-immunoprecipitation between $\alpha3$ integrin and MMP-9. This assay requires up to 1mg of protein per immunoprecipitation reaction, and thereby requires the preparation of a large amount of cell lysate. The amount of integrin stimulating antibody required to treat cells sufficient for the requisite amount of lysate was prohibitive and therefore this experiment was not performed. We had hoped that the integrin perturbing laminin peptide provided an economically viable alternate treatment for this experiment. However, recent supplies of this peptide have proved unreliable in their ability to stimulate the invasive activity of untransfected MDA-MB-231 cells. Finally, plating the wild-type c-Src transfected cells onto gelatin did not induce the MMP-9 $\alpha3$ integrin association. This was disappointing in light of recent data suggesting that plating LOX melanoma cells onto a gelatin matrix is sufficient to induce an association between another protease, seprase, and $\alpha3\beta1$ integrin (Mueller et al. 1999). These technical issues prevented us from finally addressing the exact nature of c-Src kinase activity in the regulation of the association between $\alpha3$ integrin and MMP-9.

We examined the possibility that the interaction between $\alpha3$ integrin and MMP-9 was being mediated by an increase in the levels of expression of $\alpha3$ integrin, regulated by c-Src activity. To examine this possibility we measured the levels of cell surface integrin expression by flow cytometry. We measured expression levels of all the major integrins expressed by MDA-MB-231 cells and saw no changes for $\alpha3$, $\alpha2$, αv, $\alpha6$ or $\beta1$ integrin data not shown). We also examined the possibility that increased levels of MMP-9 in the culture medium of the parental cell line were sufficient to cause the interaction between MMP-9 and $\alpha3$ integrin. We addressed this by co-culturing the parental cell line with either the activated c-Src (Y527F) or the kinase dead c-Src (K295R) transfected cells. However, there was no effect on the invasive capacity of the untransfected cell line when cultured in the presence of the c-Src (Y527F) transfected cells (data not shown). This suggests that the constitutive kinase activity of c-Src, as well as causing the transcriptional upregulation of MMP-9, is also regulating some other as yet unidentified.

component required for the interaction with α3 integrin at the cell surface. This level of regulation remains to be identified.

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Appendix 1: Key Research Accomplishments

- Identified increased invadopodia activity in activated c-Src (Y527F) transfected cells over parental, wild-type or kinase-dead c-Src (K295R) transfected cells.

- Measured increased MMP-9 expression in activated c-Src (Y527F) transfected cells over parental, wild-type or kinase-dead c-Src (K295R) transfected cells.

- Determined that MMP-9 is enriched at the invadopodia membranes of activated c-Src (Y527F) transfected cells.

- Determined that MMP-9 was responsible for the measured increase in invadopodia activity of the activated c-Src (Y527F) transfected cells over parental, wild-type or kinase-dead c-Src (K295R) transfected cells.

- Identified a cell surface interaction between MMP-9 and $\alpha 3$ integrin but not either $\alpha 2$, αv or $\alpha 6$ integrins in the activated c-Src (Y527F) transfected cells.

Appendix 2: Reportable Outcomes

Manuscripts:

Emma T. Bowden, Erik W. Thompson, Akira Myoui, Toshiyuki Yoneda and Susette C. Mueller. Activated c-Src induces increased matrix degradation at invadopodia mediated by an association between MMP-9 and $\alpha3\beta1$ integrin. Manuscript submitted to Cancer Research. (copy attached, appendix 3)

Abstracts and Presentations:
Emma T. Bowden, Erik W. Thompson and Susette C. Mueller. Activated c-Src induces increased matrix degradation at invadopodia mediated by an association between MMP-9 and $\alpha3\beta1$ integrin. Poster presented at the 13th Annual Georgetown University Student Research Days, March 1999. (copy attached, appendix 3)
PI was selected for oral presentation as a finalist in the postdoctoral category.

Emma T. Bowden, Erik W. Thompson and Susette C. Mueller. Activated c-Src induces increased matrix degradation at invadopodia mediated by an association between MMP-9 and $\alpha3\beta1$ integrin. Oral presentation at Georgetown University Student Research Days, April 1999. (copy attached, appendix 3)
PI won first place in postdoctoral category.

Emma T. Bowden, Erik W. Thompson and Susette C. Mueller. C-Src kinase activity regulates the expression of MMP-9 and its subsequent association with $\alpha3$ integrin at invadopodia. Poster presented at the 1st Annual Lombardi Cancer Center Science Fair, February 1999. (copy attached, appendix 3)
PI won first place in postdoctoral category.

Employment/ Research Opportunities:

Submitted abstract as part of a competitive application to attend training course in analytical and quantitative light microscopy at the Marine Biological Laboratory, Woods Hole, MA, April 1999.
PI was accepted to course and attended May 6th -May14[th], 1999.

PI applied for postdoctoral position with Dr. Anton Wellstein of the Lombardi Cancer Center. High profile from successful presentation of these studies resulted in PI being given a senior postdoctoral position with the opportunity to continue development towards independent research.

Appendix 3: Manuscripts and Abstracts

MMP_conv.doc

ACTIVATED C-SRC INDUCES INCREASED MATRIX DEGRADATION AT INVADOPODIA MEDIATED BY AN ASSOCIATION BETWEEN MMP-9 AND α3β1 INTEGRIN.

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Running Title: Activated c-Src induces increased matrix degradation.
Keywords: Invasion, MMP-9, MMP-2, Invadopodia, Breast Cancer.

Abstract

Overexpression of activated c-Src leads to increased invasive behavior in MDA-MB-231 cells. Localized matrix degradation and invadopodia activity was increased more than 4 fold. Zymograms to analyze conditioned media from these cells demonstrated that the level of the extracellular matrix degrading metalloproteinase, MMP-9 was dramatically up-regulated in transfected cells expressing constitutively activated c-Src. Conversely, expression levels of MMP-9 in transfected cells expressing the dominant negative, kinase inactive c-Src were suppressed. Matrix degradation could be inhibited using a monoclonal antibody that blocked the enzyme activity of MMP-9. These results suggest that membrane-associated MMP-9 was largely responsible for the localized matrix degradation observed at invadopodia in cells overexpressing kinase activated c-Src. Subcellular fractionation confirmed the localization of MMP-9 to invadopodia-enriched fractions. Interestingly, activated MMP-2 was also present in these fractions. Co-immunoprecipitation, Western blotting and zymography demonstrated that MMP-9 is localized at invadopodia via an interaction with a cell surface receptor, $\alpha 3\beta 1$ integrin. We conclude that c-Src activation promotes invasion, in part, by increasing the expression of MMP-9, which then associates with $\alpha 3\beta 1$ integrin, to promote localized matrix degradation at invadopodia.

Introduction

Tumor metastasis requires proteolytic degradation of ECM components to facilitate the local invasion of basement membranes (Liotta et al., 1991). There is significant evidence to implicate matrix metalloproteinase (MMP) family members in this process (Matrisian, 1990). Of these, there is an increasing body of literature suggesting that MMP-9 in particular, and its regulation in tumor cells, can affect invasive potential. For example, elevated levels of MMP-9 expression have been detected in a number of human cancers, including lung, bladder and breast (Rha et al., 1997; Iizasa et al., 1999; Bianco et al., 1998). *In vitro*, cellular transformation can initiate inappropriate levels of MMP-9 expression and this represents a major contribution to neoplastic transformation (Sato et al., 1993). Over-expression of MMP-9 is sufficient to confer invasive capabilities on otherwise non-invasive cells (Bernhard et al., 1994). And, conversely, it has also been demonstrated that inhibition of MMP-9 expression or activity can inhibit invasive behavior (Sehgal et al., 1998).

MMP-9 is one member of the metalloproteinase family of zinc-dependant endopeptidases. The MMPs can be divided into 3 distinct subgroups based on their substrate specificities (for review see (Birkedal-Hansen et al., 1993)). There are interstitial collagenases (MMP-1 and MMP-8), stromelysins (MMP-3 and MMP-10) and the type IV collagenases (MMP-2 and MMP-9). The substrate specificities of the MMP family members overlap. However, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are the only members to have gelatinolytic activity.

All members of the MMP family are secreted as proenzymes that require cleavage for activation (Ogata et al., 1995). This provides one checkpoint in the regulation of activity of this enzyme. It has been well documented that organo-mercurial compounds or trypsin will activate MMP-9 *in vitro* (Ogata et al., 1995). Several proteases, including MMP-3, MMP-2, plasmin and tissue kallikrein have been suggested as *in vivo* activators for MMP-9 (Fridman et al., 1995; Ogata et al., 1992; Makowski & Ramsby, 1998; Menashi et al., 1994). However, there is little evidence of an *in vivo* activator. MMP-9 activity is also controlled by the regulation of gene expression. Signaling events leading to the activation of NFκB, SP-1, Ets, AP-1 and the retinoblastoma binding factors in tumor cells can all contribute to the increased expression of MMP-9 by transcriptional

activation (Sato et al., 1993). These signals can be initiated by v-Src transformation, a number of growth factor and integrin mediated signaling pathways (Sato et al., 1993; Kondapaka et al., 1997; Ellerbroek et al., 1998; Khan & Falcone, 1997).

Integrins are transmembrane proteins consisting of α and β heterodimers. Each integrin has distinct ligand binding activity. These receptors mediate both cell adhesion and migration *in vitro* (for recent review see (Miyamoto et al., 1998)). Integrins have a critical role in transducing signals from the ECM to the inside of the cell and also regulate how those signals are interpreted within the cell. Distinct roles for integrin heterodimers have been described in invasive tumor cell behavior including modification of migratory properties and regulation of invasive capacity *per se* (Coopman et al., 1996; Lochter et al., 1999; Rabinovitz & Mercurio, 1997; Shaw et al., 1997). Since it has been suggested that uncontrolled ECM degradation could actually impede invasive behavior *in vivo*, a mechanism to coordinate cell migration and adhesion and the localization of proteolytic activity to distinct regions of the cell surface would result in directed cellular invasion (Montgomery et al., 1994; Ruoslahti, 1992). This model implicates the presence of cell surface receptors for secreted matrix degrading proteases and there is some evidence to suggest that this is the case. For example cell surface receptors for the urokinase type activator and MMP-2 have been identified (Blasi, 1993; Polette & Birembaut, 1998; Brooks et al., 1996). Evidence that integrins can act as cell membrane receptors for proteases has been described. MMP-2 and αvβ3 integrin were colocalized on angiogenic blood vessels and melanoma cells *in vivo* (Brooks et al., 1996). Furthermore, purified recombinant MMP-2 and αvβ3 were found to interact *in vitro* and binding was mediated through the C-terminus of MMP-2 (Brooks et al., 1996). To date, MMP-9 has been isolated from plasma membrane associated fractions however, a plasma membrane receptor has not been identified (Partridge et al., 1997; Toth et al., 1997).

In this study we provide evidence that kinase-activated 527-c-Src expression leads to a transcriptional upregulation of MMP-9. This is in agreement with published data where v-Src transformation upregulates MMP-9 expression. We have demonstrated in 527-c-Src transfected cells, that elevated MMP-9 expression levels are sufficient to increase invadopodia activity and localized matrix degradation. We have also identified a functional association of active MMP-9 with the plasma membrane at invadopodia. We

provide data demonstrating that both MMP-9 and activated MMP-2 are present in invadopodia enriched fractions. MMP-2 has been demonstrated as an *in vitro* activator for MMP-9 and may therefore regulate the MMP-9 mediated increase in invasion demonstrated by the kinase-active 527-c-Src overexpressing transfectants. $\alpha3\beta1$ integrin binds MMP-9 at invadopodia suggesting that this interaction recruits MMP-9 to the sites of local matrix degradation. As such, $\alpha3\beta1$ integrin represents the first cell membrane receptor for this ECM degrading protease.

Materials and Methods

Materials

      Anti metalloproteinase mouse mAbs, Ab-1 against MMP-9 and Ab-3 against MMP-2 were obtained from Oncogene Research products (Cambridge, MA). Anti-integrin antibodies, mouse mAb P1B5, against α3-integrin and mouse mAb P1E6, against α2 integrin were obtained from Collaborative Biomedical Products (Bedford, MA). The rat mAb C27 against β1 integrin was generously provided by Dr. W. T. Chen (Lombardi Cancer Center, Georgetown University, Washington, DC.). Mouse mAb L230 against αV integrin was from the American Type Culture Collection (ATCC, Rockville, MD).

Cell Culture

      HT1080 fibrosarcoma cells were obtained from the Lombardi Cancer Center Tissue Culture Shared Resource and originated from the ATCC. MDA-MB-231 cells stable transfected with c-Src constructs were the generous gift of Dr. Toshiyuki Yoneda (University of Texas Health Science Center at San Antonio, San Antonio, TX) (Myoui et al., 1996). Multiple clonal cell lines transfected with kinase activated 527-c-Src or kinase inactive (kinase-dead) 295-c-Src were examined to ensure that all exhibited similar behavior in our assays. However, data from one clone of each cell line is presented.

      Cells were maintained in Falcon flasks (Becton and Dickenson Labware, Plymouth, U.K.) in a 1:1 mixture of RPMI 1640:DMEM (Mediatech, Washington, DC) supplemented with 10% Fetal bovine serum (Hyclone Laboratories Inc., Logan, Utah), 2 mM L-glutamine and 100 units/ml penicillin/streptomycin sulfate (Gibco/BRL). Cells were grown in the presence of 7.5% CO_2 and passaged regularly to avoid post-confluent growth. Cells were examined regularly for the presence of *Mycoplasma* contamination.

Immunofluorescence Microscopy

      C-Src transfected MDA-MB-231 cells were cultured on crosslinked gelatin or fluorescein isothiocyanate (FITC)-labeled, crosslinked gelatin films. Cells (7 x 10^4 per 18mm gelatin coated coverslip) were incubated overnight at 37 °C. Cells were fixed with 3.7% formaldehyde/0.1% Triton X-100 in PBS for 10 minutes at room temperature

before permeabilization in 0.5% Triton X-100 in PBS for 5 minutes again at room temperature. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) at a final concentration of 80ng/ml. Coverslips were mounted using the ProLong antifade kit (Molecular Probes, Inc., Eugene, OR). Images of nuclei per field and degraded FITC-gelatin were digitized using Image Pro Plus from Media Cybernetics (Silver Spring, MD) *— attached 3 ar Clumpos* and a Toshiba integrating camera from I-Cube (Crofton, MD). Image analysis was *et wit* performed using the Optimas 5.2 image analysis program from the Optimas Corporation *ftc* (Bothell, WA). FITC-gelatin film degradation experiments were carried out on triplicate *filte gels.* coverslips, with at least 25 fields per coverslip (100 cells minimum) analyzed. For each field (0.01mm^2), the area of the degraded zones (μm^2) and the number of cells were summed and the areas of degraded zones per cell was calculated.

Antibody inhibition experiments were performed using the MMP-9 Ab-1 antibody from Oncogene Research products, which has previously been demonstrated to have MMP-9 enzyme activity blocking properties. An irrelevant antibody of the same isotype was used as a control for these experiments. Concentrated antibody solutions were added to both the FITC-gelatin during production of FITC-gelatin coated coverslips, as well as to the growth media after the cells were fully attached and spread. Images were captured and quantified as described above.

Total Cell Lysates, Cell Fractionation and Western Blotting

C-Src transfected MDA-MB-231 cells were cultured at 5x10^6 cells /30cm diameter plate on crosslinked gelatin films according to a previously described protocol (Mueller et al., 1992). For total lysates, cells were washed 3 times in ice-cold Tris-buffered saline (TBS) (150mM NaCl, 50mM Tris/HCL, 50mM Tris pH 7.5) and solubilized in 10mls TBS, 0.1% Triton X-100 for 30 minutes at 4°C. Gelatin and remaining cellular material were scraped from the plate dounced and solubilized for a further 30 minutes at 4°C. Centrifugation at 7,700g removed insoluble material, including gelatin and samples were taken for protein determination (bicinchoninic acid assay (BCA), Pierce, Rockford, IL).

Cells were fractionated by shearing to obtain a cell contact (CC) fraction and a cell body fraction. The cell body fraction was further separated into the cell body

membranes (CBM) and cytosol fractions (CYT) (Mueller et al., 1992). Briefly, each plate was washed in TBS. Cell bodies were sheared with a glass rod into a small volume of TBS. Triton X-100 was added to 0.1%, the cell bodies were dounced and solubilized by rotation at 4°C for 20 minutes. The invadopodial membranes, embedded in the gelatin matrix, were rinsed in TBS before being scraped up with the crosslinked gelatin into TBS/ 0.1% Triton X-100. After douncing and solubilization at 4 °C for 20 minutes, the CC fraction containing the invadopodia was centrifuged at 9,000g for 20 minutes at 4 °C to remove any insoluble material including gelatin.

Gelatin Zymography

To isolate conditioned media, cells were plated at 2.3 x 10^5 cells per 9.62 cm^2 dish and left to settle and spread in serum containing media before transfer to serum free, phenol red free IMEM containing 1mM sodium pyruvate, non-essential amino acids, trace elements and insulin/transferrin/selenium solution, all from Biofluids (Rockville, MD), 2μg/ml human fibronectin from Becton Dickinson Labware (Franklin Lakes, NJ) and vitamins from Gibco BRL (Grand Island, NY). After 2-3 days, conditioned media was spun at 7,700g for 10 minutes and frozen in aliquots at ⁻80°C. HT1080 conditioned media was concentrated for zymography using gelatin sepharose beads as described previously (Collier et al., 1988). Fractions and lysates prepared as described above were also analyzed by gelatin zymography after concentration on gelatin sepharose beads. SDS-Page gels co-polymerized with 2mg/ml gelatin were used to resolve both the latent and activated species of MMP-2 and MMP-9.

Co-immunoprecipitation

Total protein lysate (500 μg) prepared as described above, was used for each immunoprecipitation experiment. Total cell lysate was incubated with 2μg of primary antibody for 2 h at 4 °C. A 15 μl suspension of Protein A/G-Sepharose beads (Boehringer Mannheim, Indianapolis, IN) were added to the lysate and primary antibody mixture, and incubated overnight at 4 °C. Beads were washed six times in TBS/0.1% Triton X-100. For Western blotting, proteins were eluted from the beads by boiling in SDS-PAGE sample buffer under reducing conditions. Protein immuno-complexes were

separated by SDS-PAGE in 8% gels and analyzed by Western blot analysis. For zymography, beads were mixed with non-reducing sample buffer and loaded directly onto the zymogram.

Results

Over-expression of kinase activated, 527-c-Src, leads to an upregulation in expression of MMP-9

Previous work has described that v-Src transformation leads to a transcriptional upregulation of MMP-9 expression (Sato et al., 1993). Reports have suggested that increases in MMP-9 expression are sufficient to lead to an acquisition of the invasive and metastatic phenotype in certain cell types both *in vitro* and *in vivo* (Bernhard et al., 1994). We wanted to examine the possibility that overexpression of constitutively activated c-Src was causing a transcriptional upregulation of MMP-9. Gelatin zymography revealed that the parental, 295-c-Src and 527-c-Src cell lines all expressed both MMP-9 and MMP-2. However, overexpression of kinase-activated 527-c-Src led to an approximately 4 fold upregulation of expression of MMP-9 over the levels expressed in the parental cell line (Fig. 1*a* and *b*, parental and 527-c-Src, MMP-9). In contrast overexpression of kinase dead 295-c-Src did not increase expression levels of MMP-9. In fact, we even saw a slight inhibition of the levels of MMP-9 produced by the 295-c-Src cell lines (Fig. 1*a* and *b*, parental and 295-c-Src, MMP-9). This suggested that kinase activity of c-Src was responsible for the increased levels of MMP-9 expression in the 527-c-Src transfectants. Levels of MMP-2 expression remained unaltered in each of the cell lines examined (Fig. 1*a* and *b*, parental, 295-c-Src and 527-c-Src, MMP-2).

Overexpression of constitutively activated c-Src leads to increased invasive capacity.

As previously described, when invasive MDA-MB-231 breast cancer cells are cultured on FITC-gelatin, the cells produce dark holes in a background of bright fluorescent matrix. These dark holes are produced by the extension of invasive membrane protrusions from the ventral surface of the cell into the matrix beneath them. These invasive protrusions have been termed "invadopodia", and their formation requires a coordination of cytoskeletal rearrangements along with the localization and activation of certain cell surface associated proteases (Monsky et al., 1993; Monsky et al., 1994; Mueller et al., 1992; Nakahara et al., 1996).

Previously, we have compared the invasive capacity of breast cancer cells with their invadopodia activity(Coopman et al., 1998). We have also demonstrated that

behavior in this assay correlates well with invasive potential as measured in other well characterized *in vitro* invasion assays (Coopman et al., 1998). Previous data has demonstrated that overexpression of MMP-9 can be sufficient to increase measured invasive and metastatic capability both *in vivo* and *in vitro* (Bernhard et al., 1994). Therefore, we anticipated an effect in our *in vitro* invadopodia assay on the invasive capacity of the 527-c-Src cell line, but not the 295-c-Src cell line. We demonstrated that overexpression of activated 527-c-Src did in fact produce a significant increase in sites of local invasion over either the parental cell line or the kinase inactive 295-c-Src overexpressing cells (Fig. 2, parental and 527-c-Src). There were no significant differences in the invadopodia activity measured for the 295-c-Src transfectants when compared to the basal levels of invadopodia activity measured in the parental cell line (Fig. 2, parental and 295-c-Src). This suggests that, at least in this system, that increased MMP-9 expression was sufficient to increase invasive potential.

MMP-9 Activity is responsible for the increased invasive behavior measured in vitro.

Our data suggested that the approximately four-fold increase in invasive behavior measured in the 527-c-Src transfected cells corresponded to the approximately four fold increase in measured MMP-9 expression levels present in conditioned media from these cells (Fig. 1). However, c-Src activation has a number of effects including cytoskeletal rearrangements as well as modulation of protease expression levels (Charvat et al., 1998). Therefore, we wanted to further examine the possibility that the increase in MMP-9 levels was directly responsible for the observed increase in invadopodia activity. The anti-MMP-9 mAb Ab-1 has been demonstrated to have an MMP-9 activity inhibitory action (Ramos-DeSimone et al., 1993). We incorporated this antibody at increasing doses into our invadopodia assay adding it to both the growth media and the gelatin matrix (Fig 3). We demonstrated a dose dependant inhibition of matrix degradation for the 527-c-Src transfectants (Fig 3, 527-c-Src 0-100). At the highest dose of antibody examined (100μg/ml) there was a significant inhibition of invadopodia activity. However, even at the highest dose we did not successfully inhibit activity to the basal level measured in the parental cell line (Fig. 3, Parental 0-100).

Interestingly, we also saw no significant effect on the invadopodia activity of the parental cell line. This suggested that the basal level of invadopodia activity measured in these cells was probably not mediated by MMP-9. However, zymogram analysis of conditioned media from these cells had clearly demonstrated that they produced significant measurable levels of MMP-9 (Fig 1, parental, MMP-9). The other gelatinolytic enzyme that we identified in the conditioned media of the parental cell line, MMP-2, is therefore a strong candidate for the basal levels of invadopodia activity measured in the parental cell line (Fig 1, parental, MMP-2). It should also be noted that an irrelevant antibody of the same isotype as the experimental anti-MMP-9 antibody did not inhibit invadopodia activity of the 527-c-Src transfected cells (Fig. 3, Control mAb, 100).

Activated MMP-2 and MMP-9 are enriched in the Invadopodia fraction of 527-c-Src MDA-MB-231 cells

An increase in invadopodia activity mediated by increased MMP-9 suggested invadopodial localization for this protease. The antibody inhibition experiment using anti-MMP-9 antibodies to inhibit the increased invadopodia activity measured in the 527-c-Src overexpressing cells suggested that MMP-9 was present and active when associated with invadopodial membranes. We have developed a subcellular fractionation protocol that allows us to isolate an enriched invadopodial membrane fraction. We concentrated the gelatinases from each fraction using gelatin sepharose and analyzed them by gelatin zymography. Using this technique we verified that MMP-9 was enriched in the invadopodia fraction of 527-c-Src transfected cells (Fig. 4, 527-c-Src, INV). A single band of 92 kDa suggested that inactive pro-MMP-9 was present in this fraction.

We also saw an enrichment of activated MMP-2 in the invadopodia fraction of both the parental and 527-c-Src transfected cells (Fig. 4, 527-c-Src, INV). Activated MMP-2 appeared exclusively in the INV fraction of the parental cells (Fig. 4, Parental, INV). We saw some activated MMP-2 in the CBM of the 527-c-Src transfected cells (Fig. 4, 527-c-Src, CBM), However, activated MMP-2 appeared predominantly in the INV fraction (Fig. 4, 527-c-Src, INV). Pro-MMP-2 appeared in all of the fractions and lysates from these cell lines (Fig. 4, parental and 527-c-Src, Lysate, CBM and INV).

MMP-9 associates with a3 integrin only in 527-c-Src

We wanted to examine the possibility that there was a cell surface receptor for MMP-9 at invadopodia. A number of transmembrane molecules have been localized to invadopodia including several integrin subunits and the activator for MMP-2, MT1-MMP. There is precedent in the literature for interactions between integrins and proteases (Brooks et al., 1996). Therefore, we examined the possibility that one of the integrin subunits localized to invadopodia, coupled MMP-9 activity to the membrane. MMP-9 has previously been colocalized with $\beta 1$ integrin at endothelial cell contacts and isolated from shed plasma membrane vesicles enriched with this integrin (Partridge et al., 1997; Dolo et al., 1998). We have previously demonstrated the localization of $\alpha 3 \beta 1$ integrin to invadopodia in MDA-MB-231 cells (Coopman et al., 1996). This integrin heterodimer is the major one expressed in this cell type and experiments using antibodies to perturb $\alpha 3 \beta 1$ integrin in this cell line proved to modulate invasive capacity (Coopman et al., 1996).

Co-immunoprecipitation, Western blotting and zymography experiments identified an association between $\alpha 3 \beta 1$ integrin and MMP-9 in the 527-c-Src transfected cell lines (Fig.5). This association was specific to $\alpha 3$ integrin, it did not occur with $\alpha 2$ integrin (Fig. 5, Parental versus 527-c-Src, $\alpha 3$ versus $\alpha 2$ integrin). The appearance of a single band of 92 kDa indicated that MMP-9 was the inactive, pro-enzyme form of the enzyme. Plus the antibody used to probe the IP-Western blot only recognizes inactive pro-MMP-9 under these experimental conditions (Ramos-DeSimone et al., 1993). Since we also only identified a single 92 kDa band from fractionated 527-c-Src transfected cells, we were not surprised to also find only the inactive pro-enzyme in a complex with $\alpha 3 \beta 1$ integrin (Fig. 4, 527-c-Src INV). However, since we have demonstrated using a functional assay, that MMP-9 is directly responsible for the increased invadopodia activity measured in the 527-c-Src transfected cells, there is a functional significance to the association between $\alpha 3 \beta 1$ integrin and MMP-9 at invadopodia.

Discussion

We have identified a unique system to examine modulation of invadopodia activity and local invasion by proteases. We have designed a model to explain the regulation of matrix degradation in the 527-c-Src kinase activated cell lines (Fig. 6). In our system, invasive potential is measured as a function of membrane associated protease activity at invadopodia. In the untransfected, parental cell line, basal levels of invadopodia activity are MMP-2 mediated. When MMP-9 is up-regulated, as in the 527-c-Src transfectants, invasive potential increased, as measured by invadopodia activity. This regulation is mediated by a recruitment of MMP-9 to invadopodia membranes, via an association with $\alpha 3\beta 1$ integrin. We hypothesize that MMP-2 activates MMP-9 and thereby increases the potential for gelatinolytic activity at invadopodia.

After numerous repetitions of these experiments, we never observed the presence of activated MMP-9 associated with any of our lysates or fractions. This is in agreement with data from published studies where only the inactive pro-enzyme was isolated from membrane associated cell fractions (Zucker et al., 1987; Zucker et al., 1990; Toth et al., 1997). However, we have provided functional evidence that MMP-9 has activity in association with invadopodia (Figs. 1 and 3). Therefore, we hypothesize that MMP-9 is secreted, recruited to invadopodia where it is bound to $\alpha 3\beta 1$ integrin, and then activated by MMP-2 as it is released from the membrane (Fig. 6).

We suggest that in the parental cell line, MMP-2 is predominantly responsible for the basal level of measured invadopodia activity. This is supported by the antibody inhibition experiment where in the parental cell line, at the highest dose of antibody, there is no significant inhibition of the basal levels of matrix degradation (Fig. 3). It has been shown previously that MDA-MB-231 cells secrete a range of proteases (Johnson et al., 1993; Coopman et al., 1998; Gilles et al., 1998). However, since our cells are plated on gelatin, the activity that we measure at invadopodia by these assays is due solely to the gelatinases we identified by gelatin zymography, MMP-2 and MMP-9. Therefore, there is probably no contribution of other proteases in these cells to these gelatin-based *in vitro* assays.

MMP-9, as other members of the MMP family is secreted as a latent proenzyme that requires activation (Ogata et al., 1995). Thus, cleavage of MMP-9 into its

catalytically active form provides a critical point in the regulation of activity. The regulation of this process *in vivo* is not well understood. Pro-MMP-9 does not appear to be activated by a plasma membrane dependant mechanism however, several cell surface activated proteases that will activate MMP-9 *in vitro* have been identified, and include MMP-3, plasmin and MMP-2 (Fridman et al., 1995; Ogata et al., 1992; Makowski & Ramsby, 1998; Menashi et al., 1994). Previously published data described the localization of not only MMP-2 but also one of its activators, MT1-MMP to invadopodia (Nakahara et al., 1997). We noted that although MMP-2 was found in association with both CBM and INV fractions, activated MMP-2 was significantly enriched in the invadopodia fractions of both the parental and 527-c-Src transfected cells (Fig. 4, parental and 527-c-Src, INV). Therefore, since we see a clear co-localization of MMP-9 and activated MMP-2 at invadopodia in 527-c-Src transfected cells, there is a clearly the potential for MMP-2 to be involved in the activation of MMP-9 at this localization. We found it interesting that MMP-9 remained as an inactive proenzyme while in direct association with the membrane. Our functional data however, demonstrates that MMP-9 does exhibit gelatinase activity while in close proximity to invadopodial membranes. If there was significant diffusion of MMP-9 from the site of activation we would see distinct differences in the characteristics of matrix degradation produced at invadopodia. In fact, we have measured the average size of a "hole" in the matrix produced by invadopodia of both the parental and the 527-c-Src transfectants and noted that there were no significant differences other than the numbers of active sites produced (data not shown). This supports the hypothesis that although we did not isolate activated MMP-9 associated with any of our membrane fractions, it remains in a close association with sites invadopodia activity.

The idea that integrin heterodimers participate in and/ or modulate invasive capacity is not a novel one. There are multiple reports in the literature describing the role of integrin subunits in modulating the balance between adhesion and invasion (Larjava et al., 1993; Xue et al., 1997; Fukushima et al., 1998). We have provided evidence that $\alpha3\beta1$ integrin coordinates invasion at invadopodia by recruiting MMP-9 to sites of active membrane degradation. Previously, we have demonstrated that integrin-perturbing antibodies could cause increases in invasive behavior(Coopman et al., 1996). We

suggested downstream intracellular pathways, initiated by integrin signaling mediated this effect. There is ample evidence to suggest that this phenomena represents one mechanism for the regulation of cell adhesion and invasive behavior in response to ECM initiated signals. However if, as our more recent evidence suggests, integrins can also coordinate the localization and membrane association of matrix degrading proteases this represents another mechanism for the regulation of invasive behavior.

Finally, we should also speculate about the signals that initiate this whole cascade of events. The upregulation of MMP-9 in our system was a direct consequence of the presence of a constitutively activated c-Src. Therefore one would predict that those signals that lead to increased c-Src kinase activation will, as a consequence, indirectly modulate MMP-9 expression and invasive potential. There is evidence to suggest that this is in fact the case, particularly for epidermal growth factor (EGF) receptor signaling (Ellerbroek et al., 1998; Charvat et al., 1998; Kondapaka et al., 1997). However, there is also data the integrin signaling can also initiate modulation of MMP-9 expression (Khan & Falcone, 1997; Larjava et al., 1993; Wahl et al., 1993). Taken together, these data can only further stress the importance of further examination of the role of integrins in invasive behavior. One must consider both the point of view that integrins can modulate expression of proteases, and also taking into account that they also have the potential to organize these proteases at the invasive cell front, represented in our model system as the site of invadopodia activity.

Figure Legends

Figure 1. 527-c-Src overexpression upregulates MMP-9 expression. Conditioned media from equal cell numbers of parental, kinase-dead 295-c-Src and kinase-activated 527-c-Src were analyzed by gelatin zymography. (*a*) Gelatin zymography revealed the presence of gelatinolytic activity at 92 and 72 kDa. Comparison to gelatinolytic standards (HT1080 conditioned media) suggested that these bands corresponded to MMP-9 and , MMP-2 respectively. SDS-PAGE and Western blotting confirmed the identity of MMP-2 and MMP-9 (data not shown). (*b*) Quantification of the zymogram reveals an almost 4 fold increase in MMP-9 expression levels between the parental and the 527-c-Src transfected cells. There was also a significant decrease in the levels of expression of MMP-9 between the parental and the 295-c-Src transfected cells. Levels of MMP-2 expression were identical between cell lines.

Figure 2. Overexpression of kinase-activated 527-c-Src causes an increase in local invasion and invadopodia activity. Parental, kinase-activated 527-c-Src and kinase-dead 295-c-Src transfected cells were grown overnight on FITC-gelatin coated coverslips. Cells were fixed and processed for immunofluorescence microscopy. Matrix degradation was quantified by image analysis. Overexpression of 527-c-Src caused an approximately 4-fold increase in invadopodia activity over that measured in the parental cell line. Overexpression of 295-c-Src did not affect invadopodia activity as measured by this assay.

Figure 3. Inhibition of MMP-9 activity inhibits local invasion and invadopodia activity of 527-c-Src overexpressing cells. Increasing doses of the anti-MMP-9 antibody (Ab-1) added to both the gelatin and growth media of the 527-c-Src transfected cells inhibited invadopodia activity in a dose dependant manner. There was no significant effect on the basal levels of invadopodia activity produced by the parental cell line. Also, an unrelated antibody of the same isotype had no effect on the invadopodia activity of the 527-c-Src transfected cells.

Figure 4. Subcellular fractionation of 527-c-Src transfected cells revealed that MMP-9 and activated MMP-2 are associated with invadopodial membranes. After growth overnight on gelatin plates, cells were fractionated by shearing away cell bodies (CBM) to leave invadopodia (INV)embedded in the gelatin matrix. The gelatinases of total cell lysates or fractions were concentrated on gelatin sepharose before zymography. MMP-9 was associated only with the INV fraction of 527-c-Src transfectants. MMP-2 was present in lysates, CBM and INV of both the parental and 527-c-Src cells. However, MMP-2 was activated in the invadopodia fraction of both the 527-c-Src transfected and parental cell lines.

Figure 5. Co-immunoprecipitation of α3 integrin and MMP-9 from 527-c-Src transfected cells. 500μg of total cell lysate from cells grown overnight on gelatin was used in each immunoprecipitation experiments. Anti-alpha integrin subunit antibodies were used to immunoprecipitate integrin, MMP-9 complexes. Immunoprecipitation reactions were analyzed by SDS-PAGE and Western blotting. Anti-MMP-9 antibodies were used to probe western blots. MMP-9 and α3 integrin co-immunoprecipitated from the 527-c-Src transfectants but not from the parental cell line. There was no co-immunoprecipitation of MMP-9 with α2 integrin, the second most abundantly expressed α integrin subunit in these cells.

Figure 6. A model for the regulation of invadopodia activity in parental and 527-c-Src transfected cells. In the parental cell line, the basal levels of invadopodia activity are mediated by MMP-2. MT1-MMP is a transmembrane MMP family member shown to activate MMP-2. MT1-MMP represents a good candidate for MMP-2 activation in this system in that it has been demonstrated to localized to invadopodia. When MMP-9 expression is up-regulated by c-Src activity, it is recruited to invadopodial membranes. At this localization, MMP-2 can activate MMP-9 leading to enhanced local gelatinolytic degradation and therefore increased invadopodia activity.
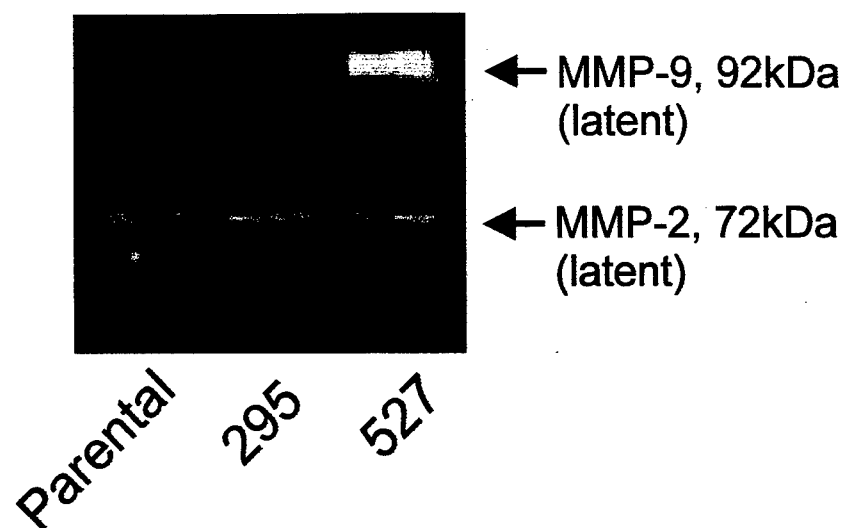
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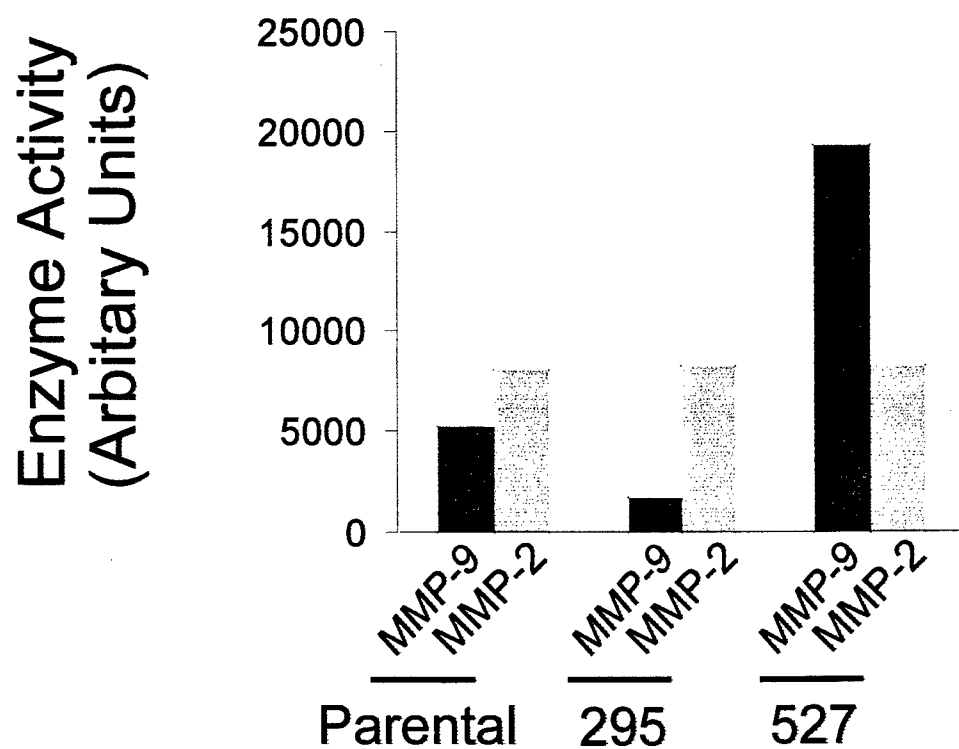
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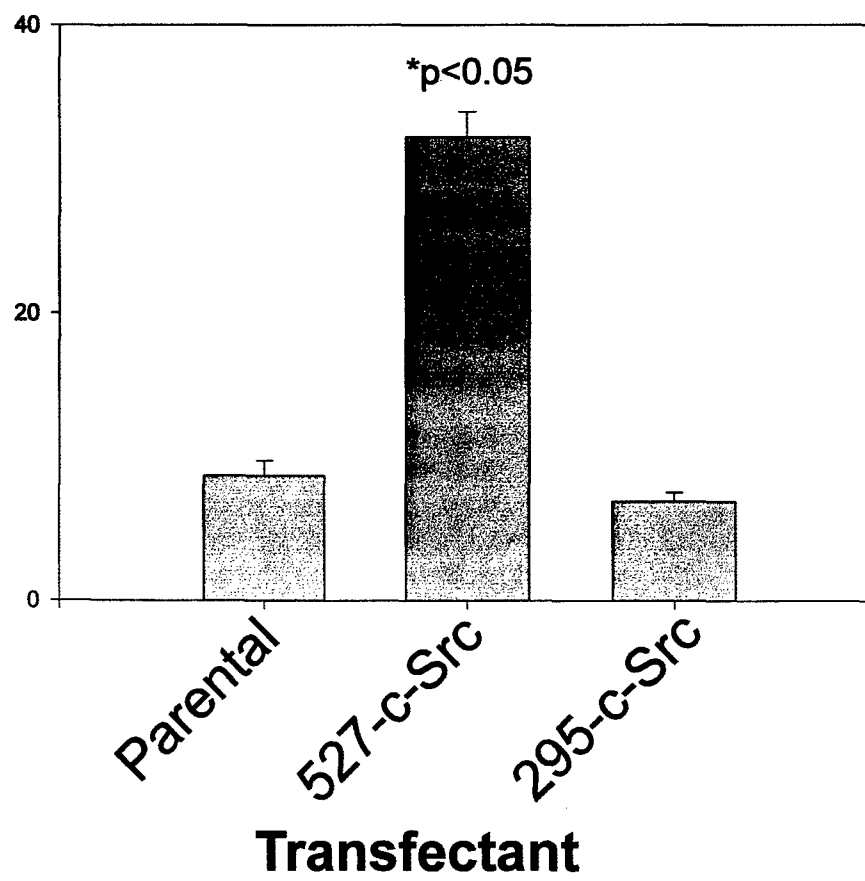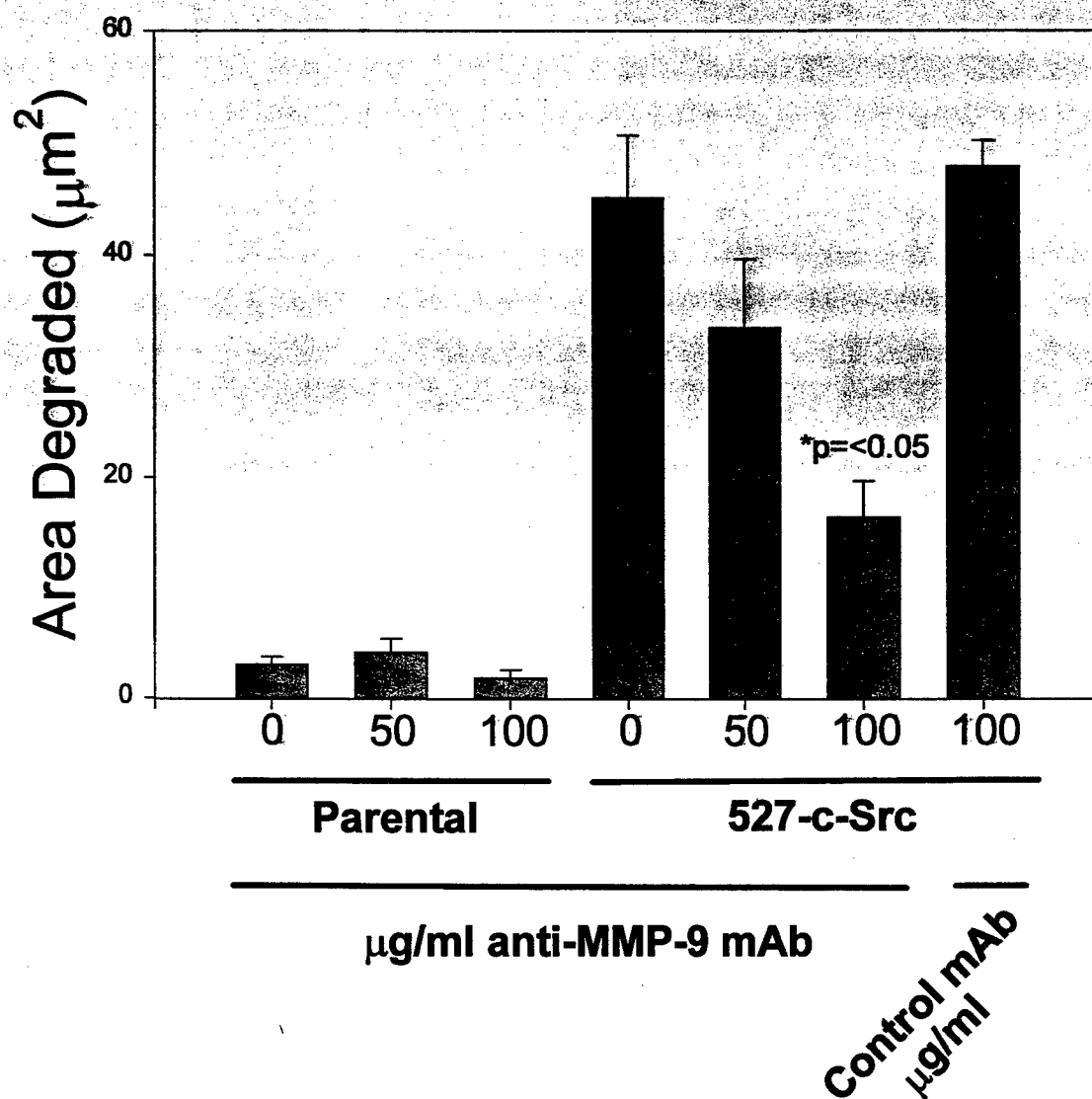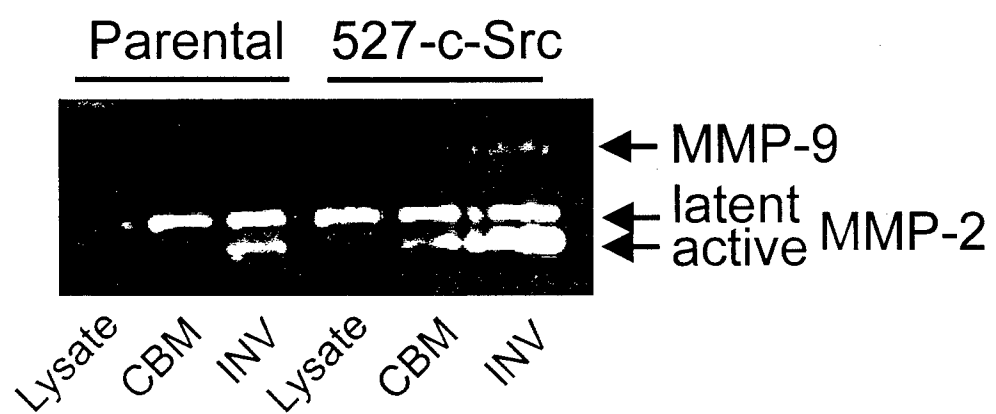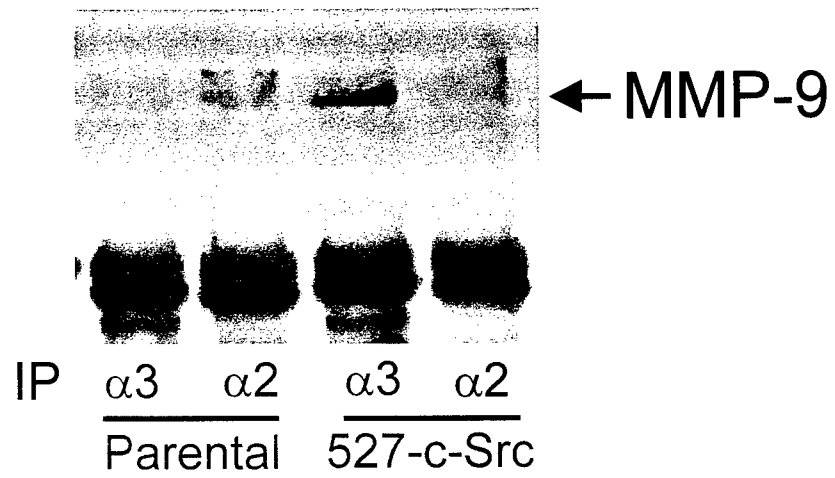
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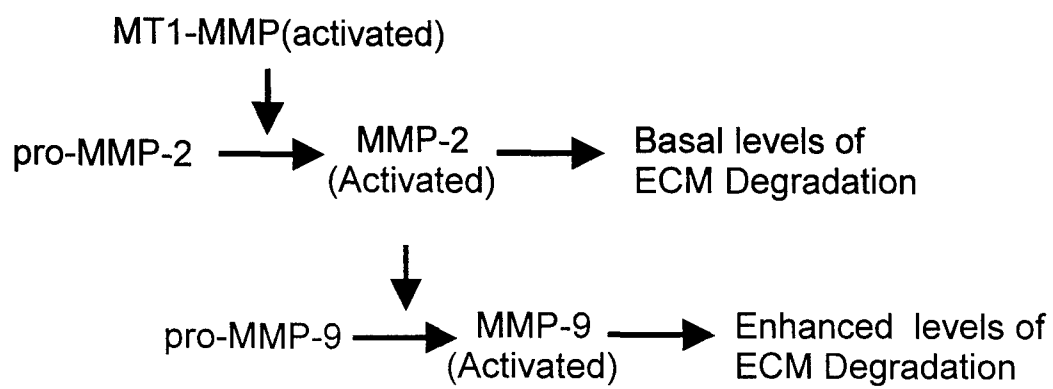
← MMP-9, 92kDa (latent)

← MMP-2, 72kDa (latent)

Parental   295   527

B.

Enzyme Activity (Arbitary Units)

MMP-9 MMP-2   MMP-9 MMP-2   MMP-9 MMP-2

Parental   295   527

Parental   527-c-Src

← MMP-9

←− latent
←− active MMP-2

Lysate CBM INV Lysate CBM INV

IP:Western Blot

← MMP-9

IP   α3   α2   α3   α2
    Parental 527-c-Src

MT1-MMP(activated)

↓

pro-MMP-2 ⟶ MMP-2 ⟶ Basal levels of
                 (Activated)   ECM Degradation

                       ↓

pro-MMP-9 ⟶ MMP-9 ⟶ Enhanced levels of
                 (Activated)   ECM Degradation

C-Src Kinase Activity Regulates the Expression of MMP-9 and its Subsequent Association with α3 Integrin at Invadopodia

Emma T. Bowden, Erik W. Thompson and Susette C. Mueller

Abstract

Invasive behavior is regulated via a concert of intracellular and extracellular signaling events. We have demonstrated that overexpression of activated c-Src leads to increased invasive behavior in MDA-MB-231 cells and that c-Src kinase activity is responsible for this phenomena. Zymograms and antibody inhibition studies revealed that our measured increase in invasive behavior could be attributed directly to increased expression and activity of the extracellular matrix degrading metalloproteinase, MMP-9. Furthermore, we have localized MMP-9 activity to invasive membrane protrusions, invadopodia. Cross-linking and co-immunoprecipitation experiments demonstrated that MMP-9 and α3 integrin co-associate in activated c-Src over-expressing cells. Together these data suggest that there is coordination of ECM degradation and integrin signaling at invadopodia that mediates cellular invasion.

ACTIVATED C-SRC INDUCES INCREASED MATRIX DEGRADATION AT INVADOPODIA MEDIATED BY AN ASSOCIATION BETWEEN MMP-9 AND α3β1 INTEGRIN.

Emma T. Bowden*, Erik W. Thompson[§] and Susette C. Mueller*

*Department of Cell Biology and Lombardi Cancer Research Center, Georgetown University.

§ Department of Medicine, University of Melbourne, Melbourne, Australia.

Abstract

Invasive behavior is regulated via a concert of intracellular and extracellular signaling events. We have demonstrated that overexpression of activated c-Src leads to increased invasive behavior in MDA-MB-231 cells and that c-Src kinase activity is responsible for this phenomena. Zymograms and antibody inhibition studies revealed that our measured increase in invasive behavior could be attributed directly to increased expression and activity of the extracellular matrix degrading metalloproteinase, MMP-9. Furthermore, we have localized MMP-9 activity to invasive membrane protrusions, invadopodia. Cross-linking and co-immunoprecipitation experiments demonstrated that MMP-9 and α3 integrin co-associate in activated c-Src over-expressing cells. Together these data suggest that there is coordination of ECM degradation and integrin signaling at invadopodia that mediates cellular invasion.

Appendix 4: Supplemental Figures

Figure 1. Western blot showing c-Src expression levels
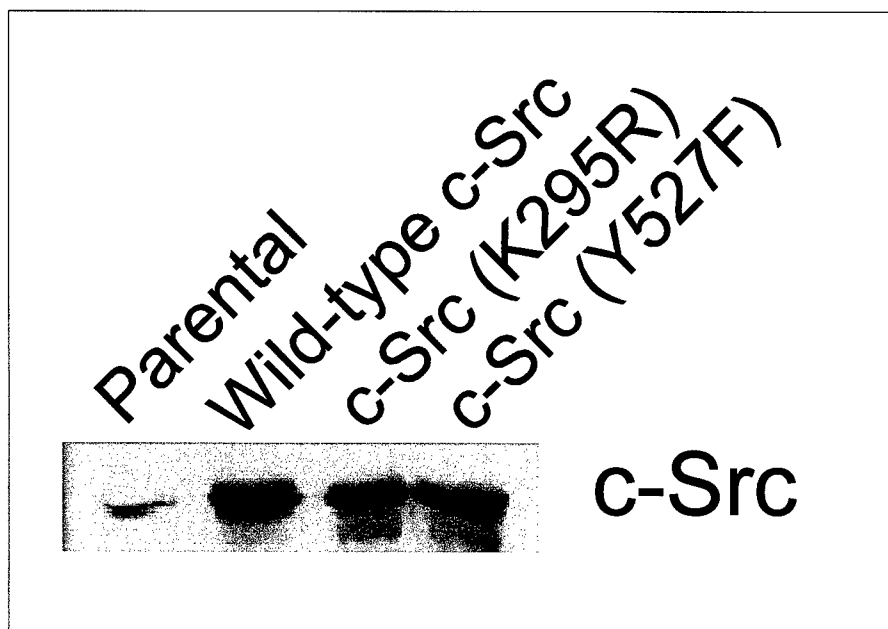
Figure 2. Western blots and immunoprecipitations demonstrate that the c-Src substrate cortactin is hyperphosphorylated in c-Src (Y527F) transfected cells
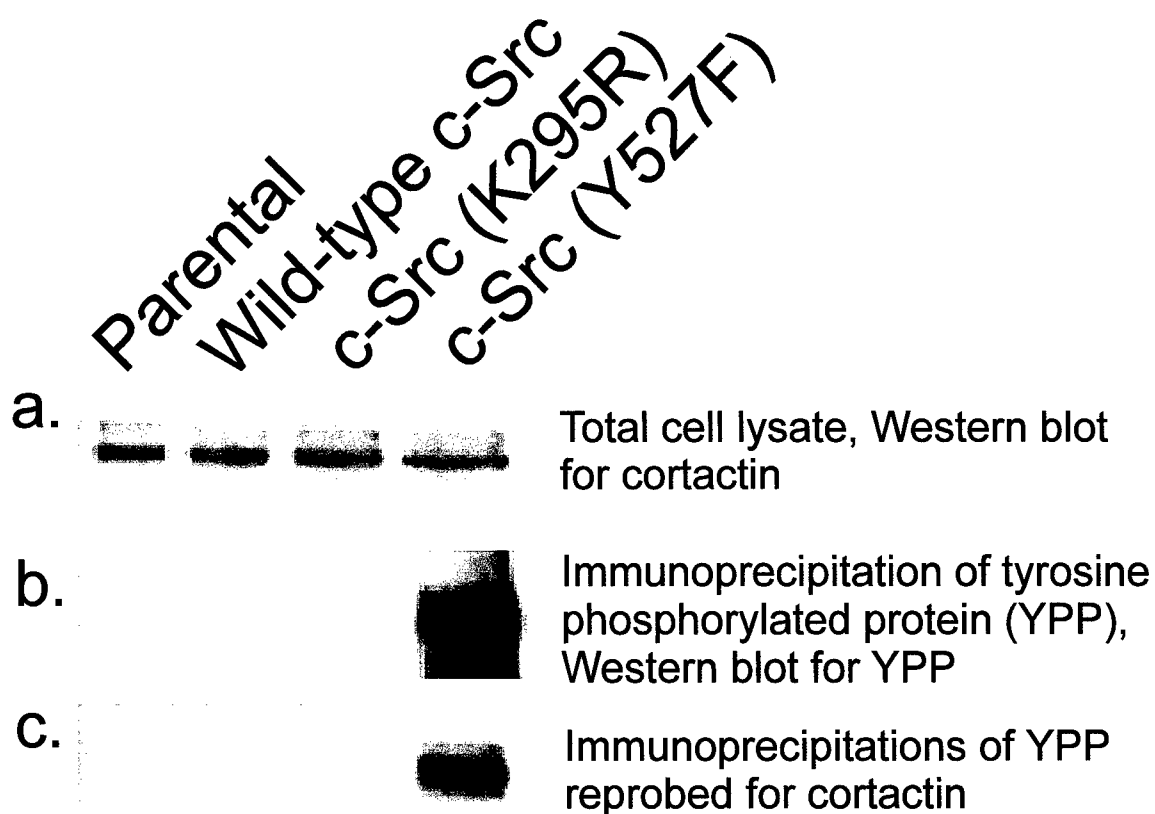

a. Total cell lysate, Western blot for cortactin

b. Immunoprecipitation of tyrosine phosphorylated protein (YPP), Western blot for YPP

c. Immunoprecipitations of YPP reprobed for cortactin

Figure 3. Overexpression of kinase activated c-Src (Y527F) causes an increase in invadopodia activity on FITC-gelatin
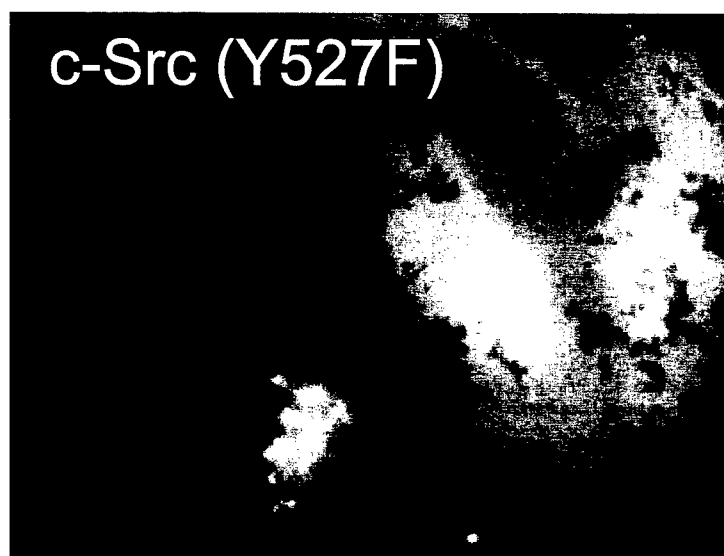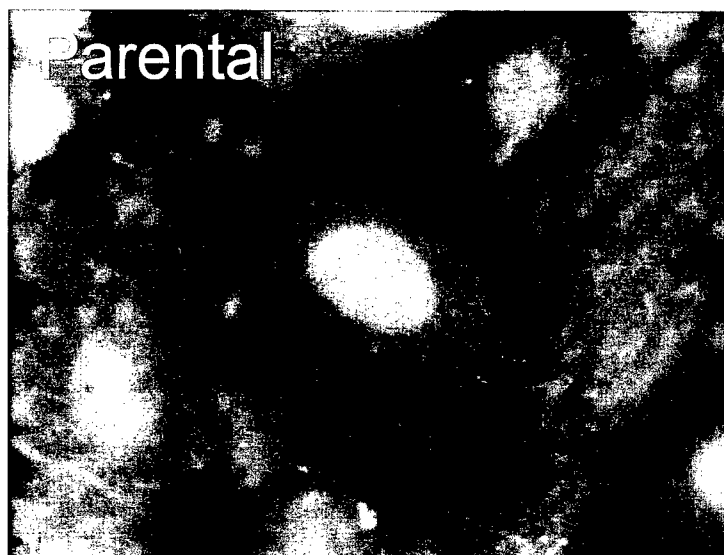
Figure 4. Dose response showing effect of anti-MMP-2 antibody Ab-3 on invadopodia activity
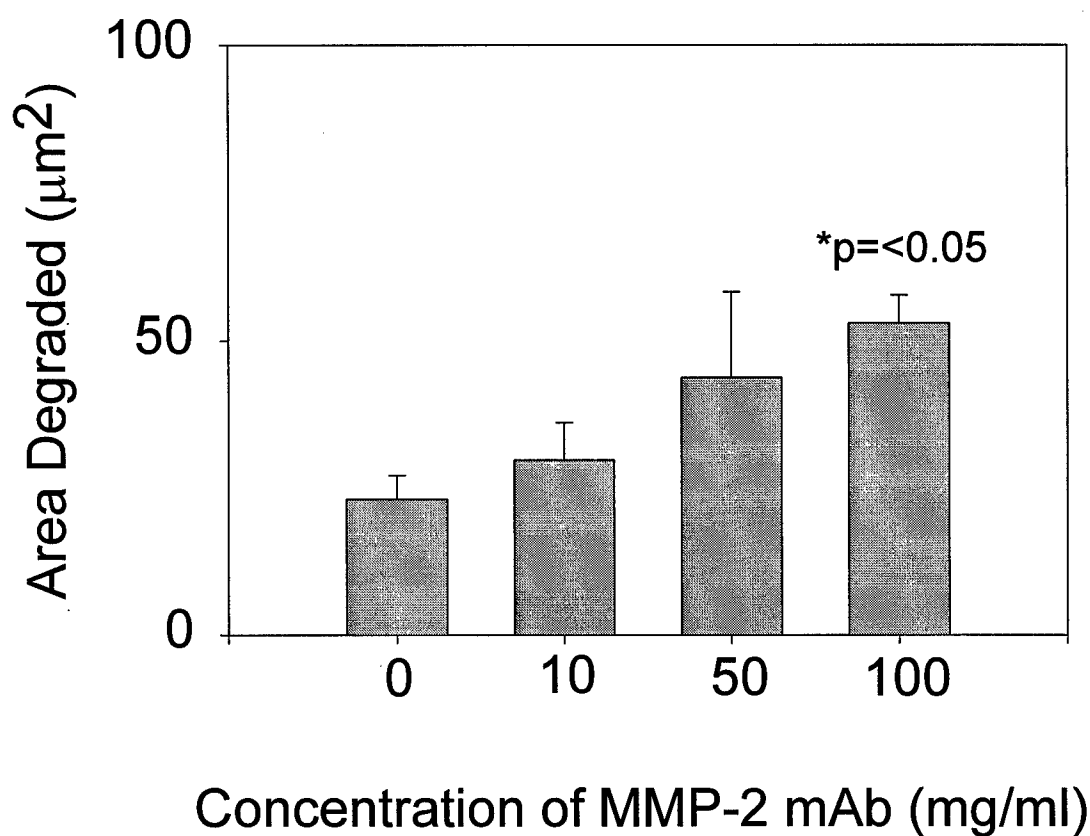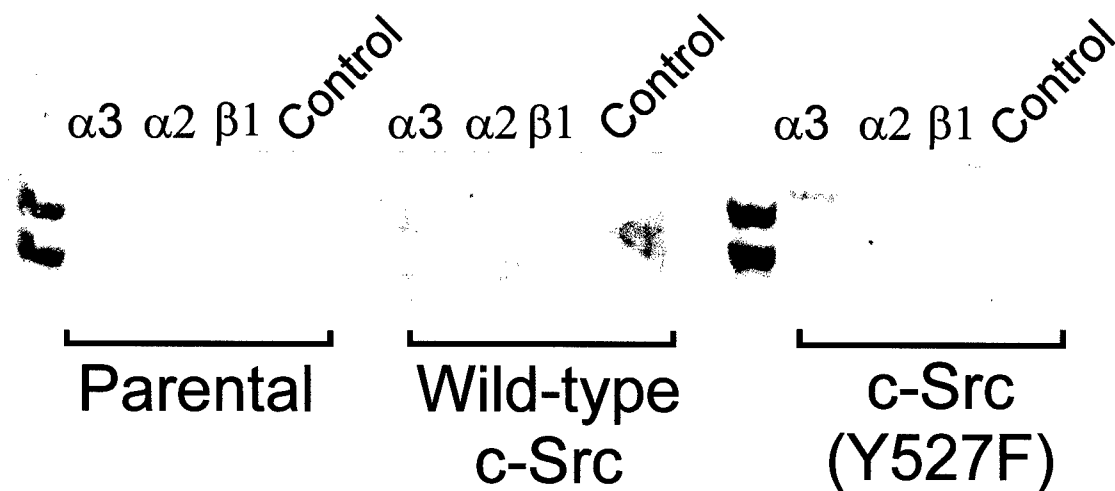
Figure 5. Integrin immunoprecipitations on a Western blot probed with anti-MMP-9 antibody

Appendix 5: Revised Abstract and Amended Statement of Work (dated 2/1/98)

PROPOSAL ABSTRACT

Invasive and metastatic progression involves extracellular matrix degradation and leads to a loss in basement membrane integrity (1). Matrix metalloproteinases (MMPs) including MMP-2 and MMP-9 have been implicated in this process (2;3;3). Both MMP-2 and MMP-9 are secreted as latent zymogens that require proteolytic cleavage for activation (4;5). MMP-2 activation appears to be mediated by membrane type MMP-1 (MT1-MMP), a transmembrane matrix metalloprotease selectively expressed by invasive human breast cancer cell (BCC) lines (6). Regulation of the activity of MMP-9 remains largely unidentified, although there is some evidence that MMP-2 is involved (7). Evidence to date suggests that both *in vitro* and *in vivo* that type I collagen stimulates invasive behavior and the activation of MT1-MMP and MMP-2 (8). These data implicate integrins known to interact with type I collagen such as $\alpha1\beta1$, $\alpha2\beta1$ and $\alpha3\beta1$ integrin. $\alpha3\beta1$ integrin is concentrated at invasive membranes called invadopodia (9). We have demonstrated in this *in vitro* model, that we can stimulate invasive behavior using anti-$\alpha3$ antibodies or laminin peptides that functionally perturb $\alpha3\beta1$ integrin molecules on the surface of MDA-MB-231 cells (9). Furthermore, the degradative activity of invadopodia is regulated by expression of MT1-MMP perhaps by regulation of MMP-2 which also localizes to invadopodia (11,12). The expression and activities of both MT1-MMP and MMP-2 are regulated by an as yet unidentified tyrosine kinase (13). We have preliminary data that in transfected breast cancer cell lines, overexpression of the c-src tyrosine kinase (WT-c-src) also leads to an upregulation of at least one other MMP, MMP-9. However, it is the overexpression of constitutively active c-src (527-c-src) that increases invasive behavior. Under these conditions, MMP-9 associates with a complex containing $\alpha3\beta1$ integrin and is implicated in the elevated matrix degradation that occurs at invadopodia. These data together corroborate the idea that $\alpha3\beta1$ integrin may be coordinating altered expression and activity of matrix metalloproteinases, thereby promoting invasive behavior at invadopodia.

Our hypothesis is functional pertubation of $\alpha3\beta1$ integrin stimulates c-src activity resulting in alterations in expression and activation of metalloproteinases and promoting invasive behavior.

STATEMENT OF WORK (18 Months)

Task 1: Identify the effects of c-src overexpression on MMP expression levels and activity. Correlate these effects with increased membrane associated matrix degradation. *(Months 1-5)*

- Quantify the *in vitro* invasive capacity of c-src-transfected and overexpressing MDA-MB-231 cells by "holes quantification" to identify the extent of membrane-associated matrix degradation at invadopodia.
- Measure the amount and activity of MMPs secreted from transfected MDA-MB-231 overexpressing c-src (WT-c-src) and constitutively activated c-src (527-c-src) using Western blotting and zymography.
- Determine the level of activated MMPs associated with the membranes of WT and 527-c-src transfected cells using zymography and/or in vitro ^{3}H-gelatin degradation assays.

Task 2. Determine the contribution/requirement of MMP-2 and MMP-9 in localized membrane-associated matrix degradation. *(Months 3-7)*

- Determine the effects of metalloproteinase inhibitors including TIMP-1 and TIMP-2 on MDA-MB-231 and c-src transfected cell lines using the "holes quantification" assay to measure invadopodia activity.
- Determine the effects of activity inhibiting, anti-MMP-9 antibodies on MDA-MB-231 and c-src transfected cell lines using the "holes quantification" assay to measure invadopodia activity.
-

Task 3: Examine the co-association of α3β1 integrin and MMP-9 in the highly invasive 527-c-src overexpressing MDA-MB-231 cells. *(Months 5-12)*

- Examine the co-association of α3β1 and MMP-9 using co-immunoprecipitation of α3β1 followed by Western blotting or zymography for detection of MMP-9.
- Determine the specificity of this association by also examining the association of MMP-2 and MMP-9 with other integrin pairs such as α2, αv, and α6β1.
- Determine the subcellular localization of the α3β1 and MMP-9 complex using subcellular fractionation and immunoprecipitation reactions.
- Determine the association of latent versus activated MMP-9 with this α3β1 integrin complex.

Task 4: Determine the role of α3β1 in c-src activation, and the subsequent upregulation of cellular invasion and MMP-9-α3β1 association. *(Months 8-18)*

- Treat WT-c-src overexpressing cells with anti-integrin antibodies, laminin peptides that bind to α3β1, and with ECM substrates such as laminin, collagen type I and fibronectin. Correlate increases in MMP-9 or MMP-2 activity with increases in invasion as measured by "holes quantification".
- Determine which of these treatments promotes the association between MMP-9 and α3β1 integrin by coimmunoprecipitation and Western blotting.
- Examine kinase inactive (295-c-src) transfected cells treated as above. Determine if kinase activity of c-src is necessary for MMP activation and MMP-9-α3β1 integrin association.

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REPLY TO
ATTENTION OF:

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MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
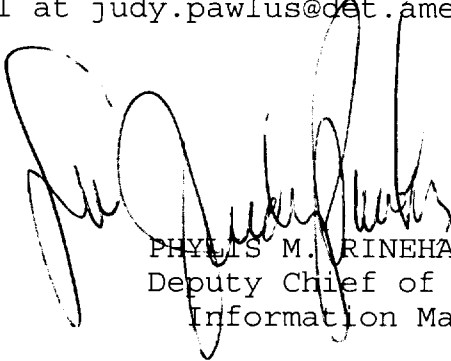VA 22060-6218

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DAMD17-96-1-6112	ADB233138
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DAMD17-96-1-6066	ADB235510
DAMD17-96-1-6029	ADB259877
DAMD17-96-1-6020	ADB244256
DAMD17-96-1-6023	ADB231769
DAMD17-94-J-4475	ADB258846
DAMD17-99-1-9048	ADB258562
DAMD17-99-1-9035	ADB261532
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DAMD17-97-1-7060	ADB257715
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DAMD17-94-J-4360	ADB220023
DAMD17-94-J-4317	ADB222726
DAMD17-94-J-4055	ADB220035
DAMD17-94-J-4112	ADB222127
DAMD17-94-J-4391	ADB219964
DAMD17-94-J-4391	ADB233754